Journal of Medical Virology 48:38–46 (1996)

Gradual Accumulation of Mutations in Precore Core Region of HBV in Patients With Chronic Active Hepatitis: Implications of Clustering Changes in a Small Region of the HBV Core Region

Gang Min Hur, Yoon Ik Lee, Dong Jin Suh, Jae Heun Lee, and Young Ik Lee

Biomedicine Research Group, Korea Research Institute of Bioscience and Biotechnology, Korea Institute of Science and Technology, Taejeon, Korea (G.M.H., Y.I.L., Y.I.L.); Department of Pharmacology, College of Medicine, Chungnam National University, Taejeon, Korea (G.M.H., J.H.L.); Department of Internal Medicine, College of Medicine, Ulsan University, Seoul, Korea (D.J.S.)

The sequence in the precore and core region of the hepatitis B virus (HBV) genome in the serum of five chronic active hepatitis patients at four different stages in each individual were studied by polymerase chain reaction and DNA sequencing to determine the prevalence and type of precore and core mutants in each chronic active hepatitis (CAH) patient. Gradual changes of the virus genome in each CAH patient in precore and core regions were identified. Except for the virus from one patient, the mutant viruses showed gradual changes of genome sequences, which resulted in the generation of stop codons at the precore and core region, causing the association of active hepatitis in each patient even in the presence of anti-HBe. Mutational hot spots in the core region, which includes a clustering of changes in a small region of 14 amino acids (codons 84–97 from the start of the core gene) were found in all patients. This region of mutational hot spots in the core might be a major target of cytotoxic T lymphocytes (CTL), which has evolved under the pressure of immune selections, and these mutants might play a important role in the pathogenesis of viral hepatitis.

KEY WORDS: hepatitis B virus, variation, polymerase chain reaction, precore/core region (pre-C/C), cytotoxic T lymphocytes (CTL)

INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic hepatic infections in man. The infection can be self-limited acute hepatitis, fulminant hepatitis, or chronic active hepatitis progressing to cirrhosis, hepatocellular carcinoma, or severe acute exacerbation with hepatic failure as well as asymptomatic healthy carriers [Brechot, 1987; Hoognagle, 1987]. During chronic hepatitis infection, however, some patients remain asymptomatic, whereas others exhibit persistent or active hepatitis. Little is known about the mechanisms and factors that play a role in viral hepatopathogenesis. Ever since there appeared some evidence that HBV-related hepatic injuries are host immune mediated [Edington and Chisari, 1975; Ferrari et al., 1987; Milich et al., 1989; Vento et al., 1985], virus antigen-specific B- and T-cell immune responses causing liver cell necrosis and direct cytopathogenicity or tumorigenicity of viral protein have been under intense investigation [Rötzschke et al., 1990; Van Bleek and Nathenson, 1990]. However, it is not clear why the clinical course is so diverse in each patient.

The C gene of HBV encodes 183–185 amino acid residues of the nucleocapsid protein and is preceded by the precore region, which starts with an initiation codon and encodes 29 amino acid residues and has been recognized as a signal sequence essential for HBeAg synthesis and secretion. The *in vivo* ocurrence of genetic HBV variants has been reported [Brunetto et al., 1991; Carman et al., 1989; Hasegawa et al., 1991; Naoumov et al., 1992]. In particular, mutations in the pre-C/C open reading frame, coding for the viral C and *e* antigens (HBcAg and HBeAg, respectively), have been demonstrated in anti-HBe-positive HBV carriers with persistent HBV multiplications [Hasegawa et al., 1991; Kaneko and Miller, 1989]. Such variants were shown to emerge spontaneously during seroconversion from HBeAg to anti-HBe and occasionally appear to emerge during interferon treatment [Raimondo et al., 1990;

Accepted for publication July 23, 1995.

Address reprint requests to Young Ik Lee, Biomedicine Research Group, Korea Research Institute of Bioscience and Biotechnology, Korea Institute of Science and Technology, Taejeon 305-606, Korea.

TABLE I. Clinical and Laboratory Data of the Patients Studied

Patients no.	Age (years)/ sex	Date at sampling	AST (IU/liter)	ALT (IU/liter)	Bilirubin (mg/dl)	HBsAg	HBeAg/anti-HBe	Liver histology
1	47/F	12 Mar, 1990	193	187	0.8	+	+/−	CAH
		7 Dec, 1990	181	162	0.8	+	+/−	CAH
		13 May, 1991	389	303	—	+	+/−	CAH
		4 Oct, 1991	85	84	1.0	+	−/+	CAH
2	18/M	11 Jan, 1989	13	22	—	+	+/−	CAH
		5 Oct, 1989	14	18	—	+	+/−	CAH
		8 Oct, 1990	297	646	2.2	+	+/−	CAH
		10 Oct, 1991	28	28	2.6	+	−/+	CAH
3	22/M	15 Dec, 1988	97	221	0.7	+	+/−	CAH
		9 Aug, 1989	104	166	—	+	+/−	CAH
		30 Jan, 1990	486	837	—	+	+/−	CAH
		10 Jan, 1991	44	46	—	+	−/+	CAH
4	26/M	21 Mar, 1987	78	236	—	+	+/−	CAH
		15 Nov, 1988	34	78	—	+	+/−	CAH
		5 Sep, 1989	351	719	—	+	+/−	CAH
		26 Mar, 1990	66	103	—	+	−/+	CAH
5	21/M	16 Oct, 1990	166	388	1.8	+	+/−	CAH
		16 Jun, 1991	237	443	—	+	+/−	CAH
		23 Nov, 1991	307	671	2.1	+	+/−	CAH
		6 May, 1992	45	75	1.3	+	−/+	CAH

Santantonio et al., 1991; Takeda et al., 1990; Thomas et al., 1991]. Although recent studies revealed that precore defective mutants, which cannot express *e* antigen found in many viremic anti-HBe-positive chronic carriers, correlated with the severity of hepatitis, the mechanisms by which the precore mutant induces severe hepatocyte injury are not known. Massive infusion of blood containing variant viruses has been identified to be associated with the development of fulminant hepatitis [Kojima et al., 1991]. The hepatitis B core antigen (HBcAg) has been postulated to be an immunological target of cytotoxic T lymphocytes (CTL) [Milich et al., 1989].

Recent results by Bertoletti et al. [1993] also suggested that residues 11–27 of the HBV nucleocapsid antigen contain a cytotoxic T-cell epitope that is recognized by CTL from all HLA-A2-positive patients with acute HBV infections. Using a panel of short peptides, the authors showed that the optimal amino acid sequence recognized by CTL is 10 mer (residues 18–27) and determined that this peptide can stimulate CTL. Since a small peptide of the core region of HBV could be recognized by CTL, it is possible that, under the selective pressure of the host's immune system, HBV variants (HBeAg-defective HBV) that have base substitutions on their CTL region become prevalent in infected patients. In this study, the nucleotide sequences of the entire precore and core region of HBV DNA was examined from chronic active hepatitis (CAH) patients in serial samples obtained at four different stages, and we analyzed whether the emergence of certain types of precore/core mutants is predictable in mutant HBVs during the course of CAH.

MATERIALS AND METHODS

Patients

Sera from five patients with CAH were taken at four different times during a 19–36 month follow-up. Liver biopsy showed CAH in all samples. All patients were tested for hepatitis B surface antigen (HBsAg), HBeAg, and anti-HBe using a commercially available RIA kit (Abbott Laboratories, North Chicago, IL). Initially, all had HBeAg, seroconverting later to anti-HBe. All patients had HBsAG and had fluctuated aminotransferase (AST, ALT) activities (Table I).

HBV DNA Purification

Isolation of HBV DNA from serum was carried out as described by Kaneko et al. [1989]. Briefly, a sample of serum was digested in 500 μl of a solution containing 250 mM NaCl, 50 mM EDTA (pH 8.0), 1.2% sodium dodecyl sulfate (SDS), and proteinase K (10 mg/ml) for 2 hr at 56°C. After two phenol-chloroform and one chloroform-isoamyl alcohol extractions, HBV DNA was ethanol precipitated and washed with 70% ethanol. The DNA pellet was recovered in 20 μl of distilled sterile water.

Amplification of the Pre-C/C and Pre-S1/S2 Region of HBV DNA by Polymerase Chain Reaction

To amplify the pre-C/C (1814–2452, 639 bp) and pre-S1/S2 gene (2848–156, 522 bp) of HBV DNA, synthetic primers were prepared according to the reported sequence of the *adr* subtype by Fujiyama et al. [1983] (Fig. 1). HBV primers for the amplification of the mutated or wild-type sequences were as follows: sense primers, P1 (nt 1774–1806, 5′-GGAGGCTGTAGGCATAAATTGGTCTGTTCACCA-3′), P4 (nt 2812–2836, 5′-GGGTCACCATATTCTTGGGAACAAG-3′); antisense primers, P2 (nt 2637–2659, 5′-GGTTAGGATAGAACCTAGCAGGC-3′), P3 (nt 2242–2263, 5′-AAAAGACACCAAATACTCAAGAA-3′), P5 (nt 178–204, 5′-CGCCTGTAACACGAGCAGGGGTCCTAG-3′. By using these primers, a segment of HBV DNA spanning the 639 bp and the 522 bp that make up the entire

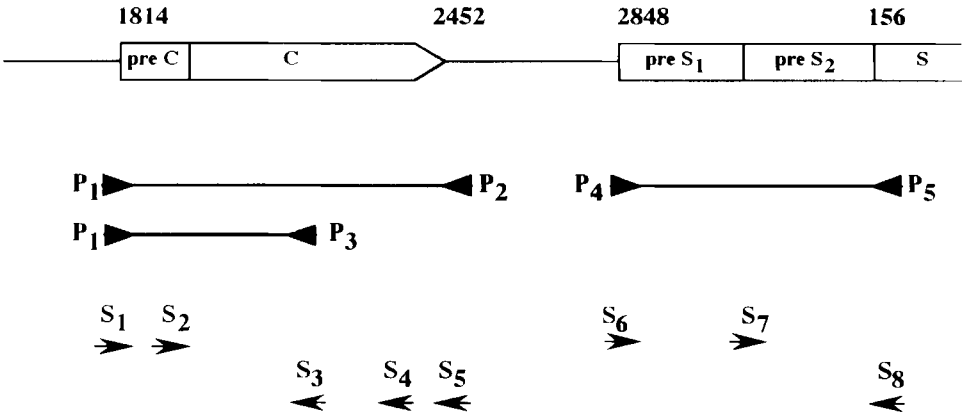


Fig. 1. Synthetic oligonucleotides used for PCR and sequencing of amplified HBV DNA and their positions on the HBV genome. Amplification primers of the pre-C/C region are denoted P1, P2, and P3, and amplification primers of the pre-S1/S2 region are denoted P4 and P5. Primers S1–S8 were used for the sequencing of pre-C/C and pre-S1/S2 regions. Nucleotide sequences of these primers are described in the text.

precore/core and pre-S1/S2 region was amplified. These primers were synthesized by the phosphoramidite method. Amplification of HBV DNA was carried out basically by using the method described previously [Saiki et al., 1988]. Briefly, 100 μl reaction mixtures containing 1 μl of the serum DNA sample, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 1 μM each of the two oligonucleotide primers, 200 μM dNTP, and 2 units of *Thermus aquaticus* DNA polymerase were overlaid with 100 μl of mineral oil. Samples were heated at 95°C for 2 min (denaturation), cooled to 50°C for 2 min (annealing), and heated to 70°C for 2 min (extension). These steps were repeated for 33 cycles in a programmable DNA thermal cycler (Hybaid). For each sample, an aliquot of the amplified DNA was fractionated by 2% NuSieve agarose (FMC BioProducts, Rockland, ME) electrophoresis and visualized by ultraviolet fluorescence after staining with 0.05% of ethidium bromide.

Subcloning and Sequencing of Amplified HBV DNA

Am amplified DNA spanning the pre-C/C and pre-S1/S2 region was inserted by blunt end ligation into the Sma I-digested plasmid PUC19 (Promega, Madison, WI), using standard cloning procedures [Sambrook et al., 1989]. Then, the recombinant plasmids were introduced into *Escherichia coli* DH-5α (BRL, Gaithersburg, MD) and cloned. To sequence the amplified segment bidirectionally, we prepared several sequencing primers: sense primers, S1 (nt 1774–1806, 5′-GGAGGCTGTAGGCATAAATTGGTCTGTTCACCA-3′), S2 (nt 1840–1864, 5′-TCTCATGTTCATGCCTACTGTTC-3′), S6 (nt 2812–2836, 5′-GGGTCACCATATTCTTGGGAACAAG-3′), S7 (nt 3178–3196, 5′-TCTAAGAGACAGTCATCCT-3′); antisense primers, S3 (nt 2242–2263, 5′-AAAAGACACCAAATACTCAAGAA-3′), S4 (nt 2323–2346, 5-TAACAACAGTAGTTTCCGGAAGT-3′), S5 (nt 2637–2659, 5′-GGTTAGGATAGAACCTAGCAGGC-3′), S8 (nt 178–204, 5′-CGCCTGTAACACGAGCAGGGGTCCTAG-3′) (Fig. 1). Three or four clones of each amplified product were sequenced according to the Sanger dideoxy method, using Sequenase version 2.0 (USB, Cleveland, OH). The reliability of the sequence results was ensured by analyzing clones from an independent amplification experiment. Sequencing products were separated on 6% acrylamide urea gel.

RESULTS

Pre-C Mutation and Formation of Termination Codon

A defective HBV with a stop codon mutation at nucleotides 1896 and 1899 of the precore region, incapable of encoding HBeAg, has been found in various HBV-related liver diseases [Raimondo et al., 1990; Standring et al., 1988]. In the present study, presence of a precore stop codon at nucleotide 1896 (28th codon from the beginning of the precore region) was analyzed to find possible sequence variations of the precore and core region of HBV in CAH patients at four different times (Fig. 2). Among five CAH patients, one had a defective precore mutation at nucleotide 1896 and one base pair deletion at nucleotide 1937 (patient 3); three others had one base pair deletion at nucleotide 1937 (patients 3, 4, and 5); (Fig. 2) All had stop codons at the stage of the seroconversion to anti-HBe (Fig. 2, Table I). Figure 3 summarizes three different cases of stop codon formations, which could be seen in five CAH patients in this analysis. These formations of termination codon by one base pair deletion at codon 12 (nt 1937) of the core gene was not identified in any of the results reported previously.

Nucleotide Sequence and Deduced Amino Acid Residue of the Precore/Core Gene

The nucleotide sequences of the variant HBV clones obtained from serum samples at four different stages of five CAH patients were compared. As is shown in Figure 2, there has been a gradual increase in nucleotide

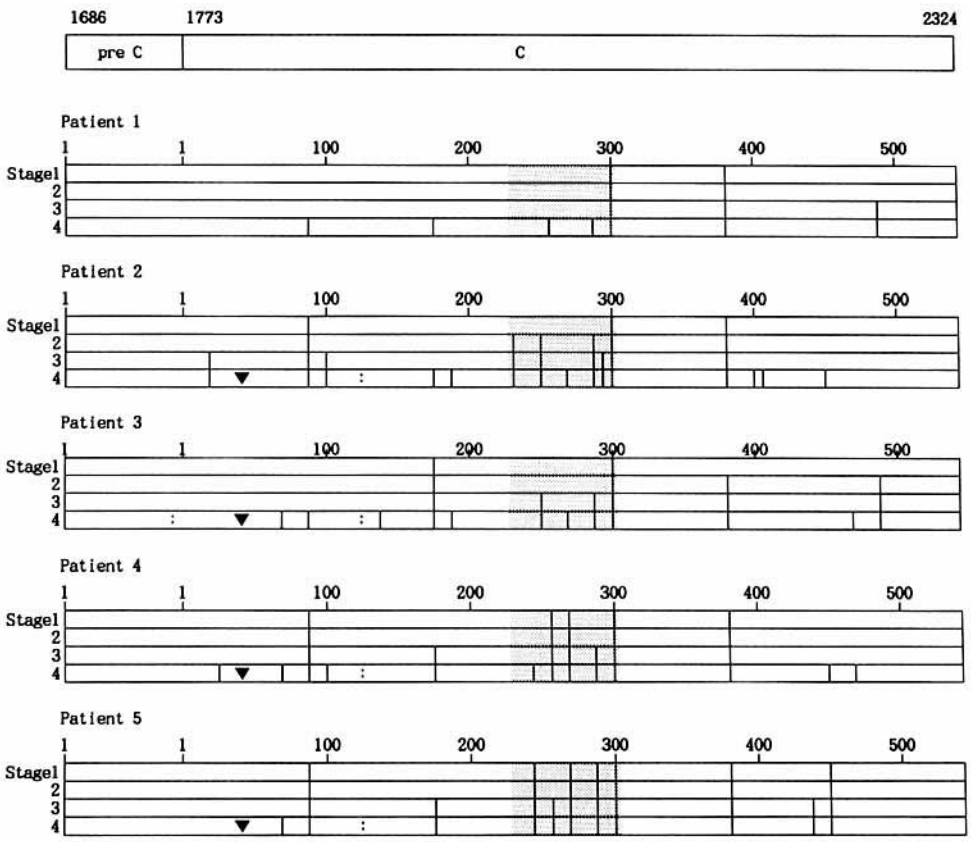


Fig. 2. Nucleotide sequences of precore and core genes in five patients with chronic active hepatitis. Only the sequences different from the wild-type *adr* subtype sequences published by Fujiyama et al. [1983] are indicated by vertical lines. The mutation clustering regions are shaded. Vertical lines, point mutations; arrowheads, one point deletion; colons, stop codon.

substitutions in different stages of CAH in each patient (Fig. 2, Table II). Except for patient 1, all other patients have a one base pair deletion at the core region (nucleotide 36, from the beginning of the core), resulting in a termination codon at the 42nd codon of core protein. As each of the patients seroconverted to anti-HBe, the number of HBV sequence variations in the precore/core region increased, and in particular these substitutions were localized within the middle portion of the core region (Fig. 2). The numbers of substitutions in different disease stages of five different CAH patients are summarized in Table IIa. In patient 1, the number of base substitutions has increased from 2 to 7 after 19 months. In patients 2–5, the numbers of base substitutions have increased from 3, 2, 5, and 7 to 15, 12, 13, and 11 after 33, 25, 36, and 19 months, respectively (Tables I, II).

The total numbers of nucleotide and amino acid changes in the precore and core regions of four different stages in five, CAH patients are summarized in Table II. As noted, the numbers of the base substitutions in stage 4 increased more than three times compared to those in stage 1 in each CAH patient. The stage 1 samples with HBeAg in the serum were detected to have 19 base substitutions; the stage 4 samples, which were seroconverted to anti-HBe, showed 62 base substitutions (Table II). As is shown in Figure 4 there has been a gradual increase in the number of amino acid changes at different stages of CAH with passing time for each patient. The locations of amino acid substitutions in the core region are shown in Figure 4. Of these 25 amino acid changes, nine amino acids (36%) were localized within a small segment of 14 amino acids (a.a. 84–97) from the start of the core gene (7.1% of the core peptide), although there are two other amino acids that showed a high frequency of mutations (amino acids 13 and 59). Sporadic base substitutions were recognized in amino acids 27, 99, 130, 149, and 158.

Clonal Analysis of Pre-S1 and Pre-S2

Mutations and deletions of the entire pre-S1 and pre-S2 nucleotide and amino acid sequence of the isolated clones against the *adr* subtype are illustrated in Figure 5 and Table III. Two defective HBVs were observed in CAH patients with anti-HBe. Among them, one had 15 base pair deletions and another had 27 base pair deletions. Such defective clones were not noted in the other three patients (Fig. 5).

Besides deletions, there were numerous silent and missense mutations of the pre-S1/S2 gene in patients

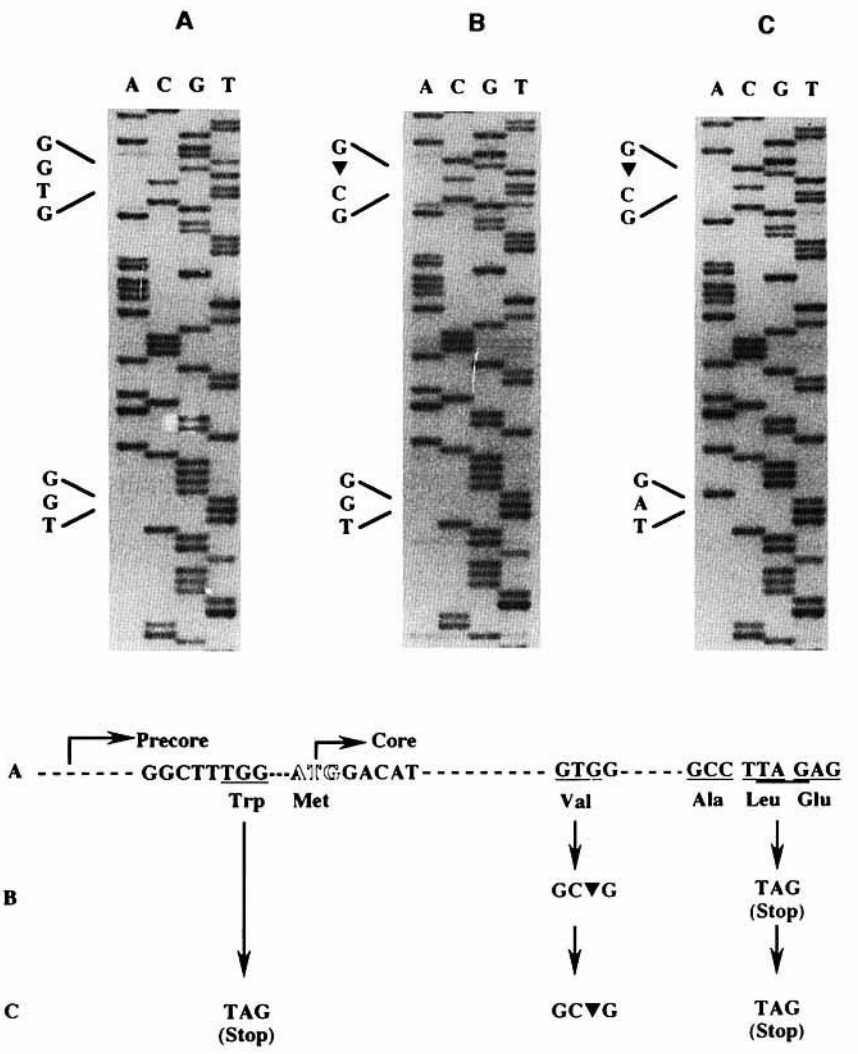


Fig. 3. Formation of termination codons in HBV DNA clones from patients with chronic active hepatitis. Representive DNA sequencing gels of three different precore/core sequences are shown at the top, and actual DNA sequences are shown at the bottom. **A**: The wild-type sequence. **B**: The core sequence with one base pair deletion (arrowheads) at nt position 1937, causing a premature stop codon. **C**: The precore sequence with a G to A mutation at nt position 1896 as well as a 1 bp deletion (arrowheads) at nt position 1937, causing a premature stop codon.

with CAH (Table III). The distributions of nucleotide substitution in the pre-S1 and pre-S2 region during the seroconversion to anti-HBe of all the samples were slightly different from those of the precore and core region. There were no significant sequential changes of deduced amino acids in a particular segment of the pre-S1 and pre-S2 region. Instead, the amino acid changes were random throughout the pre-S1/S2 region (data not shown).

DISCUSSION

Little is known about the mechanisms and factors that play a role in viral hepatopathogenesis. Previous studies suggested that the host immune response appears to play an important role in the pathogenesis of the disease [Thomas et al., 1988]. Alternatively, several recent studies suggest that different clinical courses of the disease are often attributable to genomic variants of the virus [Brunetto et al., 1991; Carman et al., 1989; Hasegawa et al., 1991; Naoumov et al., 1992]. Recently, several reports indicated that mutations in the pre-C/C open reading frame, coding for HBcAg and HBeAg, were found in fulminant hepatitis and severe chronic hepatitis [Günter et al., 1992; Hasegawa et al., 1991]. In addition, others have indicated that a variety of mutations of HBV occur in the pre-S region as well as in the pre-C/C region in the HBV carrier state.

In the present study, the pre-C/C and pre-S1/S2 genes of HBV DNA were cloned using serial serum samples from CAH patients during a 19–36 month follow-up. The findings indicate the emergence of and gradual takeover by mutated HBV sequences, with marked rearrangements of both precore/core and pre-S1/S2 cod-

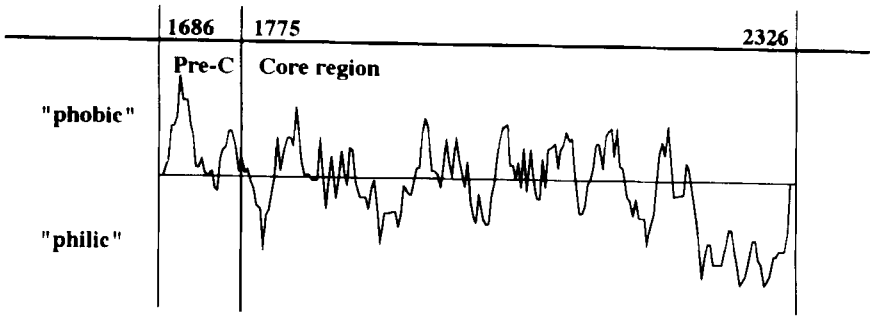
TABLE II. Numbers of Nucleotide Substitution and Amino Acid Changes in Precore and Core Gene of Four Different Stages in Five CAH Patients

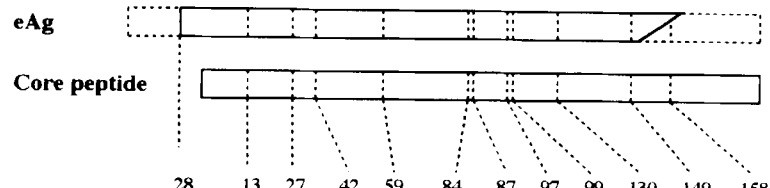
Patient[a]	Nucleotide substitution	Silent mutation	Missense mutation	Nonsense mutation	One point deletion
1					
Stage 1	2	2	—	—	—
Stage 2	2	2	—	—	—
Stage 3	3	3	—	—	—
Stage 4	7	4	3	—	—
2					
Stage 1	3	3	—	—	—
Stage 2	6	4	2	—	—
Stage 3	9	3	6	—	—
Stage 4	15	7	8	—	1
3					
Stage 1	2	2	—	—	—
Stage 2	4	4	—	—	—
Stage 3	6	4	2	—	—
Stage 4	12	8	4	1	1
4					
Stage 1	5	5	—	—	—
Stage 2	5	5	—	—	—
Stage 3	7	5	2	—	—
Stage 4	13	9	4	—	1
5					
Stage 1	7	4	3	—	—
Stage 2	7	4	3	—	—
Stage 3	10	6	4	—	—
Stage 4	11	5	6	—	1
Total					
Stage 1	19	16	3	0	0
Stage 2	24	19	5	0	0
Stage 3	34	20	14	0	0
Stage 4	62	37	24	1	4

[a]Stages 1–4 classified by sequential sampling of the five CAH patients.

ing regions when serum samples were tested at four different stages from five patients with CAH. The HBV mutant forms identified in the present study had several interesting features; first, HBV DNA from one of the patients, after seroconversion to anti-HBe, possessed a G- to A- point mutation at nucleotide 83 in the precore, region, converting codon 28 from tryptophan (TGG) to a stop codon (TAG; Fig. 3) This precore region defect was identical to that found previously in anti-HBe and HBV DNA-positive carriers [Günter et al., 1992; Naoumov et al., 1992]. However, this may not be a general feature; a base pair deletion at core codon 12, which caused a premature stop codon in the core gene, was also found in four of five CAH patients along with seroconversion to anti-HBe. No such nucleotide deletion in this region causing a termination codon at 28 has been reported previously in the *adr* subtype, or in other subtypes. Second, it had been suggested that the core peptide (HBcAg) was an immunological target of CTL in HBV infection [Ferrari et al., 1987; Milich et al., 1989]. Ehata et al. [1992] proposed that a mutation clustering region of 18 amino acid residues (codon 84–101 of the core gene) was found in 15 CAH liver disease patients infected with the *adr* subtype HBV. In this study, we found numerous silent and missense point mutations at the precore/core and pre-S1/S2 gene in patients with CAH. Such variants clustered in only a small segment of the core gene (codons 84–97).

These results are almost the same as the results of Ehata et al. [1992], who found hypervariable sequences in the core region of HBV. Therefore, it is suggested that codons 84–97 in core sequences might be a strong immunological response epitope to host immune responses. Although the core sequences of the early samples with HBeAg-positive serum had fewer changes, more clustered amino acid substitutions were found at the core codons 84–97 in the last samples, after they had seroconverted to anti-HBe. These findings imply that the mutated virus can be an escape mutant that can remain after immune clearance, because the major antigenic determinants are thought to localize within the mutation clustering region of the core peptide where the amino acid sequence is shared with HBe protein. It has been shown that the secretory HBeAg peptide is derived from the cleavage of the precore and core peptide at its amino-terminal and carboxy-terminal residues, after translocation into the endoplasmic reticulum [Standring et al., 1988]. Thus the secretory HBeAg consists of a part of the precore region and the majority of the core region. Sequential changes of nucleotides in the core region in five patients suggested that the amino acid residue change in the hypervari-





		28 W	13 V	27 I	42 L	59 I	84 L	87 S	97 I	99 Q	130 P	149 V	158 R
Stage 1 patient	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	A	-	L	-	-	I	-
Stage 2 patient	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	G	L	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	A	-	L	-	-	I	-
Stage 3 patient	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	V	-	-	-	G	L	R	T	-	G
	3	-	-	-	-	-	-	G	L	-	-	-	-
	4	-	-	-	-	-	-	-	L	-	T	-	-
	5	-	-	-	-	T	A	-	L	-	-	I	-
Stage 4 patient	1	-	-	-	-	T	-	-	L	-	-	-	G
	2	-	A	V	*	T	-	G	L	A	T	I	-
	3	*	A	-	*	T	-	G	L	-	-	-	-
	4	-	A	-	*	-	A	-	L	-	T	-	-
	5	-	A	-	*	T	A	-	L	-	T	I	-

Fig. 4. Schematic presentation of precore and core peptide with hydrophobicity profile of HBV pre-C/C region. Changes of amino acid residues different from the wild type *adr*-subtype amino acid squences are shown. Amino acid residues were numbered from start of precore or core gene. Core amino acid are numbered from the beginning of the core region. Stages 1–4 were classified by sequential sampling of five CAH patients. Amino acid residues are expressed by universal genetic codes: A, alanine; G, glycine; I, isoleucine; L, leucine; P, proline; G, glutamine; R, arginine; S, serine; W, tryptophane; *, termination codon.

able region preceded the appearance of a termination codon in the precore and core region.

Our data suggest that a virus with a mutation in the hypervariable region of the core peptide might induce a termination codon in the precore and core region to prevent further secretion of the modified HBeAg and to avoid the attack from CTL. Defective clones in the pre-S1 and pre-S2 gene were also observed in the serum, which had been seroconverted to anti-HBe, of two CAH parients. Two patients had deletions of 15–27 base pairs. Compared to the changes in the precore/core region, the nucleotide changes in the pre-S1/S2 region of variant HBV in all five patients did not have gradual nucleotide changes at four different stages (Table III). This would make it difficult to substantiate the view that the pre-S gene is under immune selection in patients with liver disease. In patients with chronic hepatitis B, envelope antigens are unlikely to be involved as targets for immune-mediated liver cell necrosis, in that expression of pre-S protein on hepatocytes in chronic HBV infection appears to reflect active viral replication and does not correlate with disease activity [Chu and Liaw, 1987; Kakumi et al., 1989]. To eliminate possible artifacts introduced by the Taq polymerase during PCR

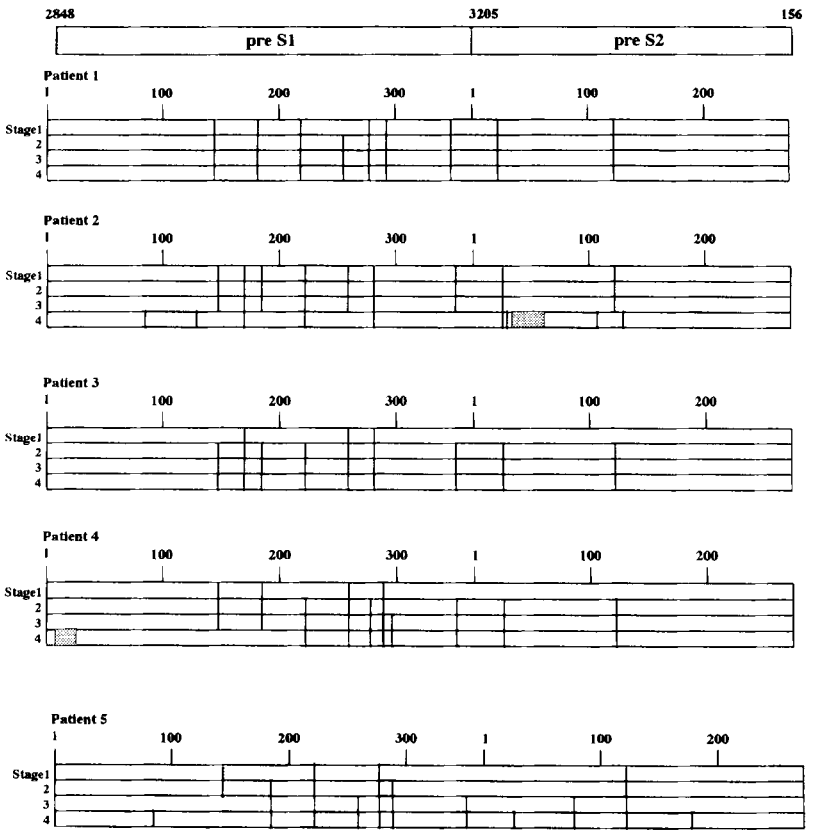


Fig. 5. Nucleotide sequences of pre-S1/pre-S2 genes in five patients from four different stages of chronic active hepatitis. Only the sequences different from the wild-type *adr* subtype are indicated by vertical lines. The deletion regions are shaded.

TABLE III. Total Numbers of Nucleotide Substitution and Amino Acid Changes in Pre-S1/S2 Gene of Four Different Stages in Five CAH Patients

Stage[a]	Nucleotide substitution	Silent mutation	Missense mutation	Deletion (codons)
1	45	24	21	0
2	62	36	26	0
3	69	40	28	0
4	69	42	26	14

[a]Stages 1–4 classified by sequential sampling of the five CAH patients.

amplification, three independent clones were sequenced from separate PCR amplifications of the HBV DNA in each patient's serum. Laboratory contamination was also ruled out by electroelution of the DNA band, which showed the expected size, and by using several sets of primers.

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